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Hypothesis: could the signalling function of membrane microdomains involve a localized transition of lipids from liquid to solid state?

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Abstract

Background: Over the past decade, it has become apparent that specialised membrane microdomains, commonly called rafts, where lipids like sphingolipids and cholesterol are arranged compactly in a liquid ordered phase are involved in cell signalling.

Hypothesis: The core of the hypothesis presented here is that resting cells may actively maintain their plasma membrane in liquid phase, corresponding to a metastable thermodynamic state. Following a physiological stimulus such as ligands binding to their membrane receptors, the tendency of membrane components to undergo a localised transition towards a gel state would increase, resulting in initial minute solid structures. These few membrane components having undergone a liquid to solid state transition, would then act as seeds for the specific recruitment of additional membrane components whose properties are compatible with the crystalline growth of these initial docks. Cells could therefore be using the propensity of lipids to assemble selectively to generate stable platforms of particular cellular components either for intra-cellular transport or for signal transduction.

Testing the hypothesis: could presumably be done via biophysical approaches such as EPR spin labelling, X-ray diffraction or FRET coupled to direct microscopic observation of cells to which very localized stimuli would be delivered.

Implications: Such a model of selective growth of membrane docks would provide an explanation for the existence of different types of microdomains, and for the fact that, depending on the state of the cells and on the procedures used to isolate them, membrane microdomains can vary greatly in their properties and composition. Ultimately, a thorough understanding of how and why lipid domains are assembled in biological membranes will be essential for many aspects of cell biology and medicine.

Background

The very existence of lipid rafts was initially proposed to explain the selective sorting of lipids and GPI-anchored molecules in polarised cells [1]. This conceptual definition has however evolved towards a more biochemical one, and the term raft is nowadays most often used to describe a fraction of cellular membranes that remain insoluble in non-ionic detergents at 4°C, have a lipid

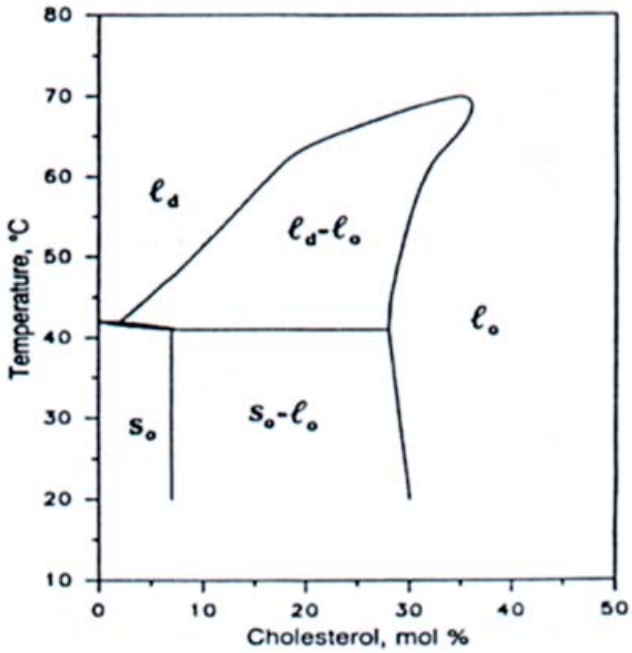

Figure 1
Theoretical phase diagram for a binary mix of a single lipid species with cholesterol (after Ipsen et al., 1987) [23]. For a thorough update on current knowledge of phase behaviour in modelled biological membranes, readers are invited to turn to the recent article by de Almeida et al. [24]. **Ld**: Liquid disorganised phase **Lo**: Liquid organised phase (because cholesterol can act as a spacer, molecules are more densely packed together, and impose more rigid conformations on one another. There is an increased thickness of the membrane, but the average speed of molecules is comparable to that seen in the Ld phase.) For both these liquid phases, the thermal energy of individual molecules is superior to the one resulting from their interaction with their neighbours. There is therefore no privileged interactions between individual molecules **So**: Solid phase. The molecules develop privileged interactions with their neighbours, resulting in stable arrangements that can lead to long lasting structures of crystalline nature.

composition rich in cholesterol and sphingolipids and display high buoyancy on sucrose gradients. A consensus about their possible size and shape has, however, proven difficult to obtain, with different techniques yielding different and sometimes contradictory results [2,3]. The latest figures derived from various optical methods seem to be converging towards a size for the core lipid rafts comprising less than ten molecules [3].

On the other hand, studies of artificially assembled membranes have clearly documented that lipids can undergo phase separations, and that cholesterol content has a critical influence on the tendency of these artificial membranes to form domains harbouring different physical states (see Fig 1 and [4] for a review). In the plasma membrane of living cells, localised modifications in lipid distribution can be observed in caveolae [2], which are invaginations involved in endocytosis. There is also a clearly demonstrated uneven distribution of various lipids between the apical and basolateral domains of epithelial cells, between the extracellular and cytoplasmic faces of the plasma membrane, and lipid clusters are believed to have a prominent role in the sorting of various lipid species and of certain proteins to specific cellular compartments [1]. It is also frequently claimed that rafts are preferentially detected at sites of focal activation, for example at the level of immunological synapses [5]. From all these examples, it is clear that, under the right conditions, lipids can partition into separate domains and/or clusters. But it is also clear that, in living cells, those separations are the result of specific mechanisms where heterogeneity between different compartments is the result of active sorting, and this must be for specific purposes.

If membrane components remain in a liquid state, I do not see how simple partitioning between ordered and disordered phases could bring either the stability or the specificity necessary for the broad scope of cellular events that biological membranes are involved in. Instead, the transition of the lipid constituents of the membrane from a liquid to a gel state would not only facilitate stable intermolecular arrangements for amplification of signals, but could also provide a mechanism for the specific recruitment of additional membrane components, or for triggering the transport of membrane components to other compartments.

Presentation of the hypothesis

The main physical basis for this hypothesis is that the transition of molecules from one state (solid, liquid or gaseous) to another one is greatly facilitated by seeding, or nucleation. A good example of this kind of phenomenon is the fact that extremely pure water freezes at a lower temperature than water containing a few impurities. This is because the impurities prime the transition of water molecules from the soluble state to that of organized ice crystals.

A more frequently encountered example of a phase transition that requires seeding to occur efficiently is that of bubbles in fizzy drinks. Bubbles form much more dramatically if champagne, for example, is poured in a dry glass than if the glass walls are wet. This appearance of bubbles along the walls of a dry glass is due to the fact that the passage of molecules from a liquid phase to a gaseous one works much better if it is primed. Dry glass contains many little scratches and specks of dust where microscopic air

bubbles are trapped, which then behave as primers for macroscopic bubble formation when the champagne is poured into the glass. Upon refilling of a glass, the microscopic bubbles will have floated away with the first round. Since priming cannot occur, the gas stays dissolved in the liquid, and the wine stays in the glass rather than frothing away.

Like specks of dust on glass walls for the formation of bubbles in champagne and like impurities for formation of ice crystals in water, I propose that, despite being thermodynamically favourable, truly organized structures in biological membranes may develop only after an initial seed has been put in place. This would represent a true transition between a liquid phase, where the membrane molecules move amongst themselves completely randomly, and a gel phase, where molecules establish lasting interactions with their direct neighbours (see Fig 1).

Many trans-membrane receptors will dimerise, or associate to co-receptors as a result of their encounter with their ligand in the extra-cellular aqueous phase. Such homo- or hetero-associations of membrane-bound components would presumably initially rely on interactions taking place outside of the membrane's hydrophobic environment. Once two membrane-bound components have become engaged in a lasting interaction within the aqueous phase, however, their respective trans-membrane domains will necessarily find themselves pushed towards a privileged relationship with one another. Even if they are not necessarily in direct contact, this situation can no longer be assimilated to a true liquid phase where components move completely independently from one another. Such a juxtaposed arrangement of two (or more) transmembrane domains may in fact represent the initial step for the transition of the lipids surrounding these transmembrane domains from a disorganised to a more organised phase. Using antibodies binding to cell-surface components is not exactly physiological, but it is a simple and efficient way to promote privileged homomeric interactions of membrane components, and the high proportions of antibodies to various surface molecules that can very efficiently trigger downstream signalling is quite remarkable (e.g. T-cell receptor, GPI-anchored molecules or simply gangliosides).

Anderson and Jacobson have previously proposed that transport of membrane-associated proteins within cells could rely on ferrying by lipid shells [2]. Inasmuch as I would be prepared to agree with the view that membrane proteins probably associate preferentially with particular subsets of membrane lipids, I would guess that, under unstimulated physiological conditions, both the proteins and the lipids would effectively remain in a liquid state, following partition coefficients, and the lifetime of such preferential associations would be very short (i.e. with off rates > 10^7 sec^{-1}). On the other hand, once multimeric protein complexes have formed, the environment resulting form the juxtaposition of several receptors may represent a very different environment favouring much more stable associations to certain lipids, and this could be providing the seed for the transition of various membrane components to a gel state. It could also be that the allosteric transition of a receptor having encountered its ligand, or the heteromeric combination of transmembrane domains of different proteins creates a micro-environment that catalyses this transition via the trapping of specific lipids. As soon as multiple molecules have started adopting a solid state, just like micro-bubbles in champagne, this minute docking area would favour the selective recruitment of additional proteins or particular lipids.

Such membrane docks are likely to adopt regular molecular arrangements typical of crystalline structures, which will in turn impose a certain specificity on the other molecules recruited to these docks, be they proteins or lipids. Crystalline growth, which is used as a purifying process in many industrial applications including metallurgy, offers much more scope for specificity than simple partition between liquid phases.

Considering the theoretical phase partition diagram shown in Fig 1, it is clear that for a transition between a liquid disordered state (Ld) and a solid one (So), a passage via a liquid ordered state (Lo) is almost mandatory. A direct consequence of this is that, in biological membranes, So areas must be preferentially surrounded by areas in Lo phase, which would explain why activated signalling molecules are preferentially associated with detergent-resistant membrane fractions when those are purified on sucrose gradients: simply because rafts (i.e. Lo domains) will probably stick to the docks (i.e. So domains), and those would then co-purify on sucrose gradients.

Most of the recent data concerning lipid rafts are based on their biochemical isolation as a fraction of membranes that remain insoluble in non-ionic detergents at 4°C and display high buoyancy on sucrose gradients. If we accept the concept of an actively maintained state of liquid phase in biological membranes, and we consider the experimental procedures used to define rafts, it is tempting to compare lipid rafts in membranes to the bubbles in fizzy drinks. Although most people would probably be prepared to swear that fizzy drinks are naturally full of bubbles, this is not quite true. The bubbles only form when you open their pressurized containers. For most of their lifetime, champagne, beer and Coca-Cola do not contain bubbles; they only start appearing when the physical conditions change, *i.e.* when the pressure drops as the

container is opened. For membrane rafts, lower temperatures and removal of the 'liquid disordered' portion of the membranes, *i.e.* the phospholipids that are soluble in non-ionic detergents, would thereby increase the portion of cholesterol and indeed promote the formation of more ordered structures (see Fig 1). But finding these structures at 4°C and/or after detergent treatment does not necessarily mean that they were present to start with in resting cells under physiological conditions, and this point of view is supported by the recent report by Heerkoltz [6] that treatment with triton can promote Lo domain formation in biological membranes. Furthermore, regarding the potential role of Lo domains under physiological conditions, the tendency of Lo areas to surround So docks spontaneously could in fact actively contribute to the further growth of the crystals, like the "oiling out" mechanism of crystallisation recently described by Bonnett and colleagues for the liquid crystallisation of a small molecule [7].

Testing the hypothesis

To test for the existence of crystalline membrane structures as a consequence of physiological stimuli, it may be possible to use X-ray diffraction. Using small-angle X-ray diffraction, cholesterol crystallites in cell membranes have recently been revealed in atherosclerotic smooth muscle cells, as well as in healthy cells of the human ocular lens, where these structures contribute to the organ's transparency [8]. Using X-ray diffraction to detect crystalline molecular arrangements that would arise transiently and focally in an isolated cell as a result of an extra-cellular stimulus may well, however, be beyond the reach of the currently available technology, and pulse EPR spin labelling or single molecule fluorescence microscopy may represent more feasible approaches to detect very small numbers of molecules switching to a solidified state [9].

As an alternative, an even more direct way would rely on direct microscopic observations. If the model proposed here is true, one could predict that the focal stimulation of a cell would result in the formation of an area preferentially enriched in components know to be recruited in microdomains, whereas remote areas in that same cell would not. This is in fact exactly what happens at the level of an immunological synapse, but the molecular mechanisms involved are far too numerous and complex to be amenable to simple experimental testing. One alternative may be to deliver a very focal stimulatory signal to a cell via the tip of a micro-injection pipet. This could for example be the release of a growth hormone for which the cell's receptor has been described to localize to rafts after stimulation. The prediction from the dock seeding model would be that molecules commonly used as raft markers such as some fluorescent lipids should concentrate around the point of the stimulus delivery. It may be that even GM1 labelled with fluorescent cholera toxin (CT) could be used for such an experiment, but one would have to bear in mind that CT has *per se* aggregating properties. If concentration of fluorescence around the area of stimulation is observed, it will be necessary to rule out the possibility of a simple coalescence of pre-existing rafts that were initially too small to detect by optical microscopy. For this, one could call upon the fluorescence resonant energy transfer (FRET) technology. If raft coalescence is occurring, the effective surface concentration between raft components will remain unchanged, and FRET signals between those components should remain comparable to those obtained on the same cell at a distance from the focus of stimulation. On the other hand, if areas of solid state form as a consequence of stimulatory signals, FRET signals between these docked components should be significantly enhanced around the focus of stimulus delivery compared to the rest of the cell. A major hurdle for the validity of such an approach, however, will be to identify fluorescent probes that could be incorporated within the assembled crystalline structures without disrupting them.

Implications of the hypothesis

In the first place, if docking areas arise in biological membranes via a process of selective crystallization, this would provide an explanation for the numerous observations that different types of membrane microdomains can exist in eukaryotic cells. It is well known that fast and slow freezing of solutions results in very different molecular arrangements because during progressive freezing crystals form preferentially between molecules that fit together well. In the same way, under physiological conditions, formation of membrane docks should occur very progressively and between similar molecules, or between specific combinations of gangliosides, other sphingolipids and sterol derivatives as well as particular transmembrane or GPI-anchored proteins.

One of the earliest observations suggesting the existence of membrane microdomains was that viral surface proteins, such as the influenza virus hemagglutinin, were incorporated into detergent-insoluble fractions as they transited through the Golgi complex [10]. What better example can there be of proteins that tend to homoassociate to form an organized structure than those viral surface molecules, which will ultimately assemble around what is effectively a microcrystal, the viral particle? Viruses may in fact be taking advantage of the tendency of biological membranes to promote homomeric crystalline arrangements for the assembling of their membranes and/or capsids, whilst excluding other cellular proteins. Results obtained by electron paramagnetic resonance (EPR) using material from influenza virus particles have in fact revealed the existence of SLOT (SLow Oxygen Transport) domains, where the 14-EASL probe was much

less mobile, and exchanged with the rest of the membrane at remarkably slow rates [11]. Binding to a solid-state dock would explain the probe's decreased mobility, whilst it's imperfect incorporation to the crystalline structures could result in the persistence of exchanges with the surrounding liquid domains.

What may be the consequences of rigid membrane structures for intracellular signalling? One of the most obvious and immediate consequences of the appearance of rigid structures within the plasma membrane would be an alteration of its impermeability [12,13], resulting in ion fluxes. In mammals, this would presumably be dominated by calcium (and sodium) penetrating inside the cell. Such fluxes may in fact represent very early steps of a signalling cascade, and would appear particularly appropriate for driving phenomena such as chemotaxis. Early in evolution, such ion fluxes may have constituted a mean for receptor-mediated signalling in unicellular organisms, before the appearance of bona-fide ion channels.

Regarding signalling pathways calling upon the recruitment of downstream signalling molecules to the receptor, one can easily envisage that the stable multi-molecular assembly of particular GPI-anchored molecules, or of certain gangliosides, could represent a seed for specific recruitment of downstream signalling molecules such as Src family kinases, heterotrimeric G proteins or Ras. This could offer a rational explanation for the observation that crosslinking GPI-linked molecules can result in specific intracellular signalling [14,15], and explain how gangliosides can act as co-receptors for FGF [16].

One further elaboration on the system comes from the recent observations that initial signaling via Fas or CD40 can trigger the release of acid sphingomyelinase from intracellular vesicles [17]. This enzyme turns sphingomyelin into ceramide, which will then favour the formation of the compacted lipid domains necessary for recruitment of additional Fas or CD40 molecules [17]. These results would in fact tie in very well with an earlier study by Massey on the effect of ceramides on the biophysical properties of bilayers reconstituted *in vitro*. This author concluded that under certain conditions, particularly at low cholesterol concentrations, the activity of sphingomyelinase could result in the appearance of gel phase areas at physiological temperatures [18].

To conclude, I would like to dwell briefly upon the concept of maintenance of the liquid state of the membrane by the cell itself. The hypothesis presented here supposes that organized, stable domain formation in biological membranes represents a sufficiently marginal thermodynamic gain to require priming for it to occur efficiently. But this also means that in this metastable situation, the formation of such structures will have a tendency to develop spontaneously, albeit much more slowly and progressively than after priming. If all this holds true, one could predict that mechanisms would exist allowing cells to actively disrupt such solid areas arising spontaneously. Proteins associating with the cytoskeleton and having an intrinsic affinity for microdomains arising spontaneously could have just such a role, much like a stirrer can prevent water from freezing below 0°C. Conversely, when the development of those docks is not spontaneous, but driven by physiological stimuli such as receptor multimerisation, the same stirring mechanisms could have a role in driving the recruitment of additional components and in bringing surrounding domains in Lo state to the signalling platform, as has been documented for cells of the immune system in several reports (see [19] for review). Once docks have truly formed, they will represent thermodynamically stable structures that would probably be very difficult to disrupt. After membrane docks have formed and duly performed their function, their persistence at the cell surface would presumably become undesirable, and particular mechanisms probably exist allowing cells to eliminate such structures actively and rapidly. The very localised and transient existence of such membrane structures having adopted a gel phase could in fact be the main reason for their having escaped detection despite intense interest in this field of investigation.

For a cell to get rid of crystallised membrane docks, I see three obvious solutions:

- *Shedding*: For certain cell types, the direct disposal of such undesirable membrane areas into the extra cellular milieu may well be the preferred route.

- *Endocytosis*: For other cell types, such an answer may be unsuitable, either because it would degrade their direct environment, or because it would be too metabolically costly. Such cells would therefore internalize the areas harbouring a solid state, and, in the cell types expressing caveolin, this may well be one of the main roles of caveolae, the flask shaped membrane structures that are enriched in membrane components of high hydrophobicity [2]. After all the components that can be recovered and recycled have been extracted by the cell's own machinery in the endosomal pathway, it can be expected that certain components of the docks' structure may remain in particularly stable arrangements, thus precluding their disassembly. Such structures, possibly in the form of minute vesicles, would then presumably have either to remain inside the cell, or be secreted by exocytosis, to be cleaned up by scavenger cells such as macrophages, that would be better equipped enzymatically for their disposal.

It seems that exosomes [20] would be very good potential candidates for the products of the two types of excretory phenomena described above.

- *Trogocytosis* (From *trogo*, to nibble in ancient Greek.): Corresponding to the active capture of membrane fragments by another cell. This phenomenon seems to occur very broadly among cells of the immune system. Indeed, after the formation of an immune synapse, lymphocytes will extract a significant portion of the components of the plasma membrane of the other cell that was involved in the formation of that synapse [21,22]. As was seen above, pathogens such as viruses would rely on membrane docks for their assembly, and the immune system could have adapted to recognise the assembly of such structures as danger signals. By nibbling rigid areas from the surface of other cells, lymphocytes, and possibly other leukocyte types, may not only be surveying their neighbouring cells for the development of dangerous pathogens, but may also have an important role in the refuse disposal of membrane docks that may be unwanted at the surface of resting healthy cells.

Abreviations used

FRET: fluorescence resonant energy transfer, EPR: Electronic Paramagnetic Resonance.

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